INFECTION AND IMMUNITY, May 2008, p. 2080–2089 Vol. 76, No. 5
0019-9567/08/$08.00+0 doi:10.1128/IAI.01428-07

Interleukin-1β Modulates Proinflammatory Cytokine Production in Human Epithelial Cells[▽]

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Received 24 October 2007/Returned for modification 29 November 2007/Accepted 26 February 2008

Periodontitis is a chronic human inflammatory disease initiated and sustained by dental plaque microorganisms. A major contributing pathogen is *Porphyromonas gingivalis*, a gram-negative bacterium recognized by Toll-like receptor 2 (TLR2) and TLR4, which are expressed by human gingival epithelial cells (HGECs). However, it is still unclear how these cells respond to *P. gingivalis* and initiate inflammatory and immune responses. We have reported previously that HGECs produce a wide range of proinflammatory cytokines, including interleukin-6 (IL-6), IL-8, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor alpha (TNF-α), and IL-1β. In this study, we show that IL-1β has a special role in the modulation of other inflammatory cytokines in HGECs challenged with *P. gingivalis*. Our results show that the increased production of IL-1β correlates with the cell surface expression of TLR4, and more specifically, TLR4-normal HGECs produce fourfold more IL-1β than do TLR4-deficient HGECs after challenge. Moreover, blocking the IL-1β receptor greatly reduces the production of "secondary" proinflammatory cytokines such as IL-8 or IL-6. Our data indicate that the induction of IL-1β plays an important role in mediating the release of other proinflammatory cytokines from primary human epithelial cells following challenge with *P. gingivalis*, and this process may be an inflammatory enhancement mechanism adopted by epithelial cells.

The etiology of periodontal disease is complex, and variance in the human host response to microbial plaque occurs and relates to the host's innate, inflammatory, or immune defense system. It is estimated that over 500 different bacterial species are capable of colonizing the oral cavity; several of these are considered periodontal pathogens and include the gram-negative bacterium *Porphyromonas gingivalis* (4, 5, 19). The recognition of invariant pathogen molecular structures, termed microbe-associated molecular patterns, is mediated by a set of gene-encoded receptors that are referred to as pattern recognition receptors, which include Toll-like receptors (TLRs). The binding of bacterial products to TLRs results in the activation of signaling molecules that eventually trigger host responses, such as the release of antimicrobial peptides, proinflammatory cytokines, and chemokines (1).

It has previously been shown that *P. gingivalis* is recognized by TLR4 and TLR2 (7) and interacts with gingival epithelial cells (16). We have shown that the activation of TLRs by *P. gingivalis* induces the release of proinflammatory cytokines from gingival epithelial cells (9). Therefore, it might be expected that a reduction in cellular TLR4 is associated with a reduction in multiple "normal" host responses and, thus, increased susceptibility to chronic inflammatory diseases, including periodontal disease. The response of the innate system in detecting pathogens and triggering the adaptive immune system is crucial. For example, neutrophil recruitment to the site of infection occurs through a chemotactic gradient, including CXC chemokine ligand 8 or interleukin-8 (IL-8), mainly secreted by epithelial cells (15); other proinflammatory cytokines, including IL-6 and tumor necrosis factor alpha (TNF-α) are also secreted by epithelial cells (9). Among the many cytokines implicated (9, 15), IL-1β appears to play a special role in inflammatory modulation.

IL-1β is a potent inflammatory cytokine involved in many important cellular functions, such as proliferation, activation, and differentiation (30), and is an important component of the innate immune response (8). IL-1β also induces the chemotaxis of leukocytes by inducing the induction of IL-8 and activating neutrophils for phagocytosis, degranulation, and oxidative burst activity (6). The release of IL-1β is a critical step in inflammation through the induction of other inflammatory cytokines (27). The induction of IL-1β is highly controlled in two stages, (i) the production of proinflammatory IL-1β (pro-IL-1β) after the triggering of TLRs and other cellular receptors and (ii) the cleavage of the precursor pro-IL-1β to mature IL-1β by caspase-1 (24). IL-1β can stimulate the cells producing it in an autocrine fashion through its own receptor, IL-1R (18, 23). It has previously been demonstrated that blocking the IL-1β receptor with anti-IL-1R results in reduced neutrophil recruitment; however, blocking TNF-α did not (22). Here we report that the induced release of IL-1β by *P. gingivalis* then modulated the production of other inflammatory cytokines, including IL-8, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α, in human primary gingival epithelial cells (HGECs) (TLR4-normal cells). We also demonstrate that this response is attenuated in TLR4-deficient epithelial cells or when TLR4, IL-1R, or caspase-1 activities were inhibited.

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▽ Published ahead of print on 10 March 2008.

MATERIALS AND METHODS

Cell isolation and culture. With University of Louisville institutional review board approval, we obtained primary HGECs from healthy patients after third-molar extractions. The gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C, and HGECs were isolated as described previously (21). The cell suspension was centrifuged at 120 × *g* for 5 min, and the pellet was suspended in complete medium, i.e., keratinocyte-serum-free medium (K-SFM; Invitrogen, Carlsbad, CA) containing 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM of 2-mercaptoethanol, 10 μM of 2-aminoethanol, 10 mM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 U/ml of penicillin-streptomycin, and 50 ng/ml of Fungizone. The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen and incubated in 5% CO_2 and 95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured. Normal human bronchial epithelial (NHBE) cells were obtained from Cambrex (Walkersville, MD), and all experiments with NHBE cells were conducted between passages 4 and 5. NHBE cells were grown in medium containing 1 g/liter (5.5 mM) glucose and formulated with bovine pituitary extract, recombinant human epidermal growth factor, hydrocortisone, insulin, epinephrine, tri-iodothyronine, transferrin, gentamicin, amphotericin B, and retinoic acid (BEGM with SingleQuots; Cambrex, Walkersville, MD). However, the cells were challenged with *P. gingivalis* in the same medium which was used for HGECs.

Bacteria. *Actinobacillus actinomycetemcomitans* strain Y4 and *P. gingivalis* strain 33277 were kindly provided by D. Demuth, School of Dentistry, Louisville, KY. *P. gingivalis* was grown at 37°C in Trypticase soy broth supplemented with 1 g of yeast extract, 5 mg of hemin, and 1 mg of menadione per liter under anaerobic conditions of 85% N_2, 10% H_2, and 5% CO_2 for 2 days. *A. actinomycetemcomitans* strain Y4 was cultured under microaerophilic conditions at 37°C in brain heart infusion (BHI; Difco) medium supplemented with 40 mg of $NaHCO_3$ per liter. Based on a previously calibrated optical density of 1 to determine the proper multiplicity of infection (MOI) and plating experiments to determine viability, we were confident that our bacterial cultures were live and within the log phase. After cultivation, the bacteria were harvested by centrifugation, washed three times in phosphate-buffered saline, and heat inactivated as described previously (15).

Cytokine induction assay. Primary HGEC cultures at the fourth passage were harvested, seeded at a density of 0.5×10^5 cells per six-well culture plate (coated with type I collagen), and maintained in 2 ml of K-SFM medium. When they reached confluence, the cells were washed twice with plain K-SFM (no antibiotics or additives) and 2 ml of complete medium was added. When they reached 90 to 100% confluence, the cells were challenged with *P. gingivalis* (MOI, 100) or *A. actinomycetemcomitans* (MOI, 100) at 37°C, 5% CO_2, for 24 h. In a blocking assay, caspase-1 was inhibited with YVAD-cmk (1 μM; Clontech, Mountain View, CA) for 1 h prior to challenge in epithelial cells treated with Pam3CSK4, an agonist for TLR1/TLR2 (1 μg/ml; InvivoGen); FSL-1, an agonist for TLR2/TLR6 (1 μg/ml; InvivoGen); R837, an agonist for TLR7/TLR8 (5 μg/ml; Invivogen); human recombinant IL-1β (0.2 ng/ml; R&D); or *P. gingivalis* for 24 h to demonstrate that the production of proinflammatory cytokines is induced by a variety of TLRs and thus is IL-1β modulated. The IL-1β receptor was inhibited with anti-human IL-1R1 antibody or its isotype control immunoglobulin G (IgG) (1 μg/ml; R&D, Minneapolis, MN) prior to 1 h of challenge. In order to determine whether there was any influence of IL-8 on IL-1β production, the cells were challenged with *P. gingivalis* in the presence or absence of an IL-8-neutralizing antibody (0.2 μg/ml; R&D). Cell culture supernatants were assayed by Luminex 100 technology using a multiplex of five cytokines, including IL-6, IL-8, GM-CSF, and TNF-α (Upstate, NY). IL-1β was assayed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's protocol.

Real-time PCR. Total RNA was extracted from cultured cells by using TRIzol (Invitrogen, Carlsbad, CA) and quantified by spectrometry at 260 and 280 nm. Ten micrograms from each RNA extract was used to perform first-strand cDNA synthesis using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) in a total volume of 100 μl. Real-time PCR was performed by using 100 ng of cDNA with an ABI 7500 system (Applied Biosystems). TaqMan probes, sense and antisense primers for gene expression of human TLR4, IL-1β, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as an endogenous control, were purchased from Applied Biosystems. We used a universal PCR master mix (Applied Biosystems) to carry out the reactions according to the manufacturer's protocol.

Inhibition of TLR4 expression. Primary epithelial cultures at the fourth passage were harvested, seeded at a density of 0.5×10^5 cells per six-well culture plate (coated with type I collagen), and maintained in 2 ml of medium until they reached 50 to 70% confluence. The epithelial cells were transfected with 100 pmol of short interfering TLR4 (siTLR4; Dharmacon, CA) or short interfering laminin (siLaminin; Dharmacon, CA) as an assay control. Briefly, 3 μl of the transfection reagent FuGENE 6 (Roche Applied Science, Indianapolis, IN) was diluted using 95 μl of serum-free medium and then 100 pmol of siTLR4 or siLaminin was added and incubated at room temperature for 15 min. The reaction was performed overnight, and the medium was replaced with fresh medium. The cytokine induction assay was performed after 48 h as mentioned above.

Flow cytometry. Human gingival epithelial cells were washed three times with phosphate-buffered saline, and 1 million cells were stained with 1 μg/ml of phycoerythrin conjugated to anti-human TLR4 or to an isotype control, mouse IgG2a (eBioscience, San Diego, CA), in 100 μl total staining buffer. The cells were analyzed by flow cytometry using BD FACSCalibur and CellQuest software.

RESULTS

Human gingival epithelial cell cultures and TLR4 expression. It has previously been shown that TLR4 deficiencies may detrimentally reduce the innate immune response to pathogenic bacteria (15). We have a bank of more than 40 primary cultures of HGECs and have determined their TLR4 levels by fluorescence-activated cell sorter analysis in both the resting and *P. gingivalis*-stimulated states. So far, we have detected three HGECs with reduced TLR4 expression at the protein and mRNA levels; these cells are defined as TLR4 deficient in both basal and challenged states. These TLR4-deficient cells are typically, but not exclusively, related to carriage of the Asp299Gly polymorphism in the TLR4 gene (15). We also utilized three different HGECs, defined as TLR4-normal cells. These cells induced the expression of TLR4 following challenge with *P. gingivalis*. Those cells are harvested from subjects free of systemic diseases and chronic periodontitis. For our experiments, we chose HGEC-9, -11, and -12, (which are primary HGEC cultures with deficiencies in TLR4 expression) and HGEC-2, -5, and -6 (with normal TLR4 expression). *P. gingivalis* was chosen for the microbial challenge, as it is considered a putative pathogen in periodontal disease and displays interesting signaling mechanisms through TLR2 and TLR4 (7) and the HGECs thus produce a wide range of cytokine responses to *P. gingivalis* (15).

Recognition of pathogenic microorganisms by the innate immune system is crucial for inflammation and to trigger the adaptive immune system to eliminate bacteria (12). We determined TLR4 gene expression in three TLR4-normal HGECs and three TLR4-deficient HGECs after challenge with *P. gingivalis*. TLR4 expression was down-regulated in the TLR4-deficient cells (HGEC-9, -11, and -12) compared to the expression in the TLR4-normal gingival epithelial cells (HGEC-2, -5, and -6) at the gene level (Fig. 1A). We also confirmed our results with protein quantification by flow cytometry. We found that TLR4 expression was down-regulated in HGEC-9 (Fig. 1B) challenged with *P. gingivalis* compared to that in HGEC-5 (Fig. 1C). We found that the other TLR4-normal (HGEC-2 and -6) and TLR4-deficient cell cultures (HGEC-11 and -12) also yielded results similar to those of HGEC-5 and -9, respectively (data not shown).

IL-1β induction in HGECs challenged by *P. gingivalis*. IL-1β is a potent proinflammatory cytokine and is produced in a variety of cells, including monocytes, following microbial perturbation (17, 27). Therefore, we examined phenotypic differences of TLR4-normal and TLR4-deficient gingival epithelial

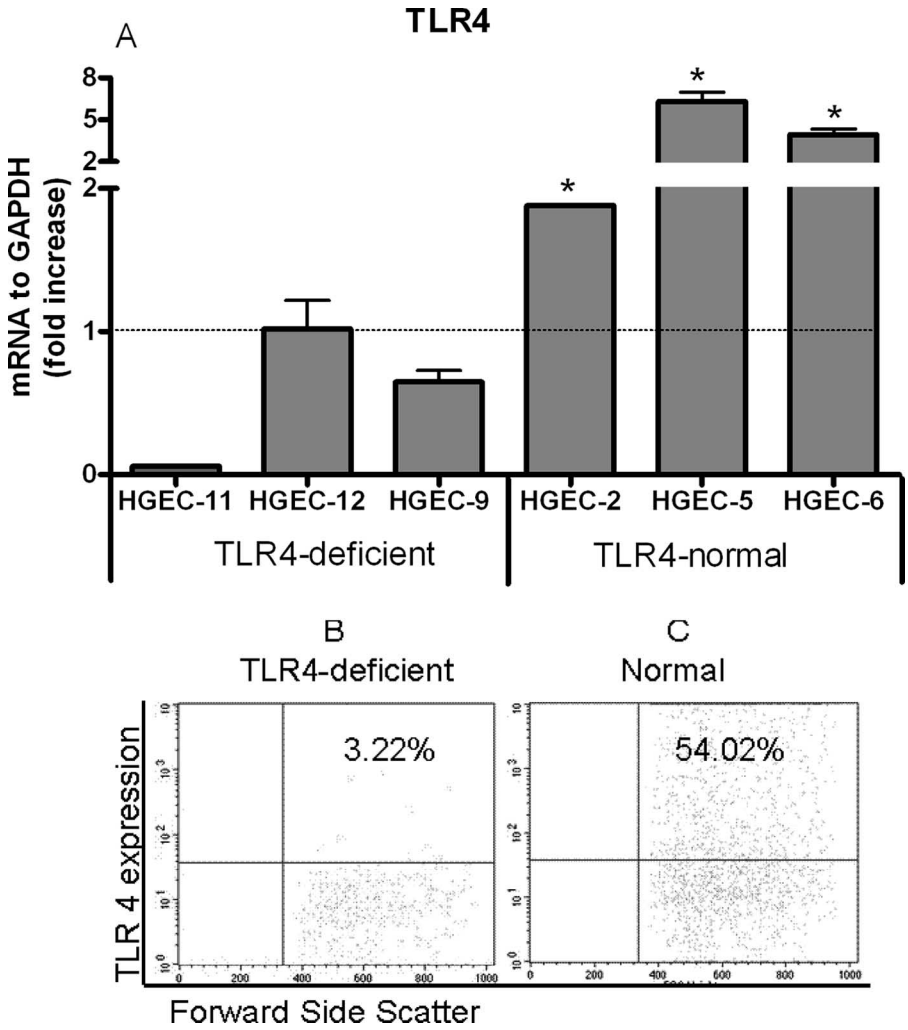

FIG. 1. TLR4 expression in human primary gingival epithelial cells. (A) TLR4-normal (HGEC-2, -5, and -6) and TLR4-deficient (HGEC-9, -11, and -12) epithelial cells were challenged with *P. gingivalis* for 24 h at 37°C. Real-time PCR was performed with an ABI 7500 system (Applied Biosystems). TaqMan probes and sense and antisense primers for gene expression of human TLR4 were purchased from Applied Biosystems, along with probes and primers for the human endogenous control GAPDH. Using a universal PCR master mix (Applied Biosystems), we carried out the reactions according to the manufacturer's protocol. The ratio of TLR4 was normalized to GAPDH mRNA. TLR4-deficient (B) and TLR4-normal (C) cells were stained with phycoerythrin-conjugated monoclonal anti-human TLR4 for 20 min at 4°C. The stained cells were analyzed by flow cytometry using BD FACSCalibur and CellQuest software. Data are presented as the means ± standard deviations (bars) of triplicate determinations. Statistically significant ($P < 0.05$) induced expression of TLR4 is indicated by asterisks.

cells to check the level of IL-1β by challenge with *P. gingivalis* for 24 h. The transcription level of IL-1β was increased more than fivefold in TLR4-normal cells challenged by *P. gingivalis* but not in TLR4-deficient cells (Fig. 2A). We next confirmed our results with protein quantification in both cell types following challenge with *P. gingivalis*. IL-1β production was slightly but not significantly increased in TLR4-deficient cells following challenge with *P. gingivalis* after 24 h. In contrast, TLR4-normal epithelial cells increased the production of IL-1β compared to the production with medium (Fig. 2B). Subsequently, we challenged both cell types with another oral gram-negative pathogenic bacterium, *A. actinomycetemcomitans*, which has previously been shown to be recognized by TLR4 (11). The production of IL-1β was significantly increased in TLR4-normal cells but not in TLR4-deficient cells (Fig. 2C). We found that all TLR4-normal cell cultures induced IL-1β production when they were challenged with either *A. actinomycetemcomitans* or *P. gingivalis*, but TLR4-deficient cells did not (data not shown). Together, our data show that reduced IL-1β secretion in TLR4-deficient cells is not exclusive to *P. gingivalis*, as IL-1β is also responsive to *A. actinomycetemcomitans* stimuli.

Proinflammatory cytokine production was attenuated in TLR4-deficient cells. Furthermore, we examined whether IL-1β was the only cytokine reduced or whether other proinflammatory cytokines, including IL-6, IL-8, TNF-α, and GM-CSF, were also decreased in TLR4-deficient cells compared to the level in normal cells following challenge with *P. gingivalis*. To address this point, TLR4-normal and TLR4-deficient epithelial cells were challenged with *P. gingivalis* for 24 h. The production of GM-CSF was minimally increased by treatment with *P. gingivalis* in TLR4-deficient cells. However, its induction was dra-

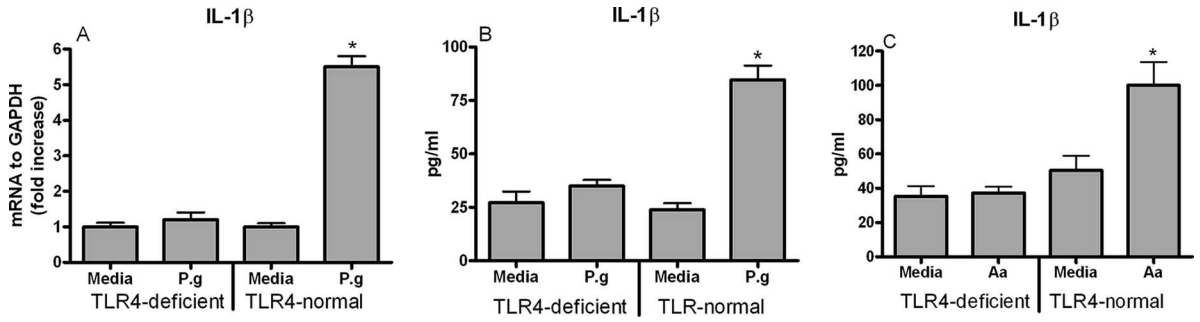

FIG. 2. IL-1β production is induced in TLR4-normal cells. TLR4-normal and TLR4-deficient epithelial cells were challenged with *P. gingivalis* (P.g) for 24 h at 37°C. (A) Expression of IL-1β was determined by real-time PCR performed with an ABI 7500 system (Applied Biosystems) in TLR4-normal and TLR4-deficient cells following normalization of the ratio of IL-1β to GAPDH mRNA. (B) Production of IL-1β was assayed with ELISA in both cell types. (C) Both cell types were challenged with *A. actinomycetemcomitans* (Aa) for 24 h, and the induction of IL-1β was detected by ELISA. Data are presented as the means ± standard deviations (bars) of triplicate determinations. Statistically significant ($P < 0.05$) induction of cytokine release and gene expression are indicated by asterisks.

matically induced in TLR4-normal cells following treatment with *P. gingivalis* for 24 h (Fig. 3A). The induction of IL-8, IL-6, and TNF-α also was significantly up-regulated in TLR4-normal cells challenged with *P. gingivalis*; however, TLR4-deficient epithelial cells did not up-regulate the production of IL-8 (Fig. 3B), IL-6 (Fig. 3C), and TNF-α (Fig. 3D) after stimulation with *P. gingivalis*. We also found that the production of IL-6, IL-8, GM-CSF, and TNF-α was induced in TLR4-normal cells but not in TLR4-deficient cells following treatment with *A. actinomycetemcomitans* (data not shown). Together, our results demonstrate that the production of not only IL-1β but also other inflammatory cytokines, including GM-CSF, IL-8, IL-6, and TNF-α, was attenuated in TLR4-deficient cells following challenge with *P. gingivalis*.

We then determined whether TLR4-deficient cells responded normally to other stimuli, including a TLR2 agonist and exogenous human recombinant IL-1β, since it has been demonstrated that *P. gingivalis* is recognized by TLR4 and TLR2 (7) and the receptor for IL-1β (IL-1R1) shares structural and signaling pathway similarities with Toll-like receptors (28). Therefore, we challenged both cell types with FSL-1 (a TLR2 agonist) or IL-1β for 24 h. The production of IL-8

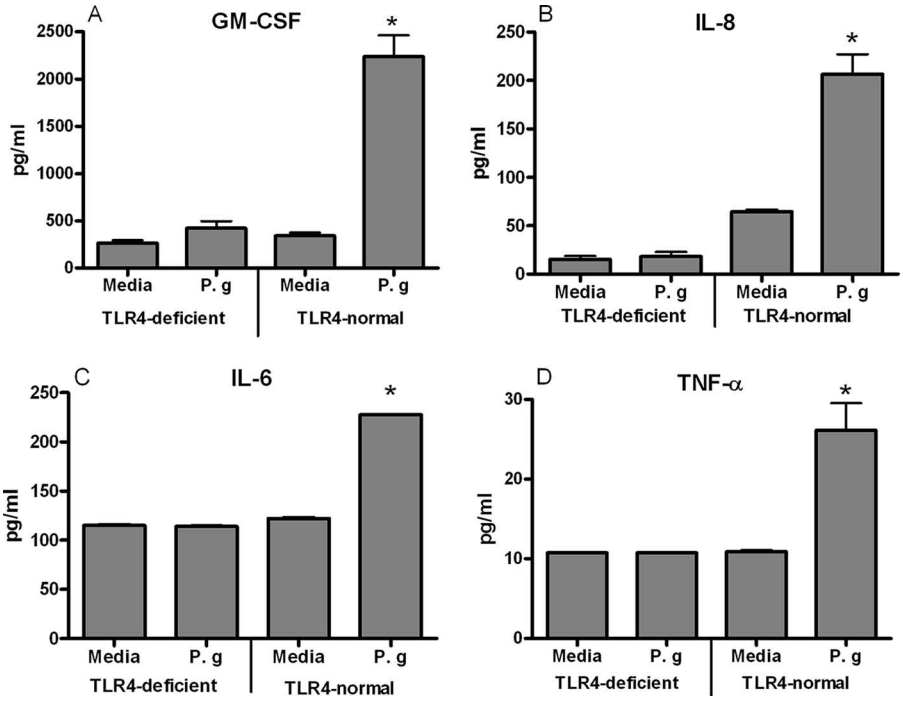

FIG. 3. Cytokine induction in epithelial cells challenged with *P. gingivalis*. TLR4-normal and TLR4-deficient gingival epithelial cells were challenged with *P. gingivalis* (P.g) for 24 h at 37°C. The production levels of GM-CSF (A), IL-8 (B), IL-6 (C), and TNF-α (D) were determined in culture supernatants by Luminex 100 technology (Upstate, NY). Data are presented as the means ± standard deviations (bars) of triplicate determinations from one of three independent sets of experiments that yielded similar findings. Statistically significant ($P < 0.05$) induction of cytokine release in the cells is indicated by asterisks.

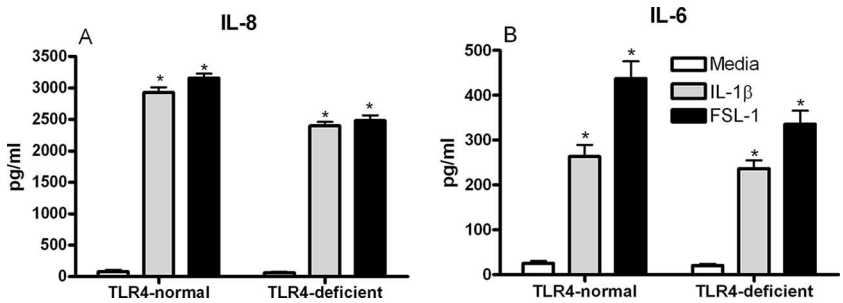

FIG. 4. TLR4-deficient cells gave normal responses to IL-1β or a TLR2 agonist. TLR4-normal and TLR4-deficient gingival epithelial cells were challenged with human recombinant IL-1β (5 ng/ml), a TLR2 agonist (1 μg/ml, FSL-1), or medium only for 24 h at 37°C. The production of IL-8 (A) and IL-6 (B) was determined in culture supernatants by Luminex 100 technology (Upstate, NY). Data are presented as the means ± standard deviations (bars) of triplicate determinations from one of three independent sets of experiments that yielded similar findings. Statistically significant ($P < 0.05$) induction of cytokine release is indicated by asterisks.

(Fig. 4A) and IL-6 (Fig. 4B) was markedly induced in both TLR4-deficient and TLR4-normal epithelial cells challenged with FSL-1 or IL-1β. We also found that other cytokines, including GM-CSF and TNF-α, were increased in both cell types after treatment with FSL-1 or IL-1β (data not shown). Together, our results indicate that reduced cytokine production in TLR4-deficient cells is not due to a lack of TLR2 signaling.

TLR4 regulates proinflammatory cytokine production in HGECs. TLR signaling activation is a critical step in inducing proinflammatory cytokines, and TLR4 is involved in *P. gingivalis* recognition (7). To examine the importance of the TLR4 receptor in the HGEC response to *P. gingivalis* and to confirm the results shown above, TLR4-normal cells were transfected with short interfering RNA (siRNA) to TLR4, TLR2, or an irrelevant gene (laminin). Initially, we determined the expression of TLR4 and TLR2 following transfection with siTLR2 or siTLR4. In a comparison with control gene (laminin) silencing, TLR4 expression was significantly down-regulated in the cells transfected with siTLR4 after stimulation with *P. gingivalis* for 24 h (Fig. 5A). In a manner similar to that for TLR4 expression, TLR2 expression was also reduced by transfection with siRNA to TLR2 in the cells challenged with *P. gingivalis* (Fig. 5B). Next, we determined proinflammatory cytokine production in the cells challenged with *P. gingivalis* following transfection with siRNA to TLR4 or TLR2. The production of IL-1β (Fig. 5C), TNF-α (Fig. 5D), IL-6 (Fig. 5E), and IL-8 (Fig. 5F) was significantly reduced after attenuation of TLR4 expression. We found that knocking in TLR4 to the cells rescued the reduced inflammatory cytokine induction in TLR4-deficient cells challenged with *P. gingivalis* (data not shown). More interestingly, the attenuation of TLR2 expression by

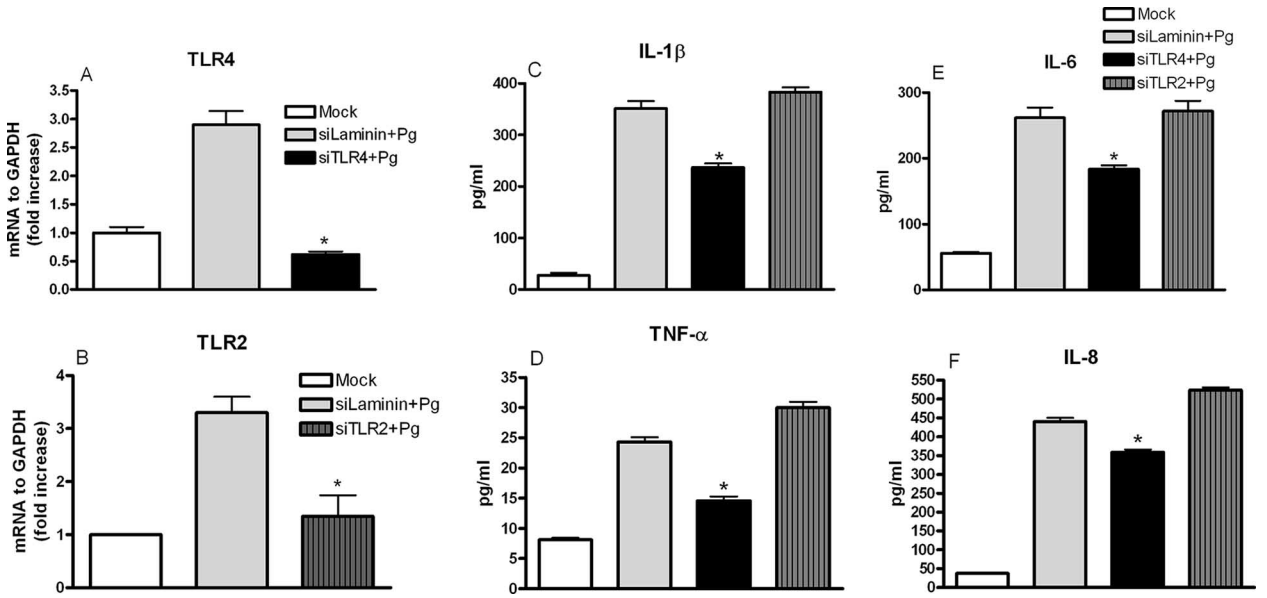

FIG. 5. Knocking down of TLR4 resulted in reduced inflammatory cytokine production. TLR4-normal epithelial cells were knocked down by RNA interference for siTLR4, siTLR2, or an irrelevant gene, siLaminin. Forty-eight hours after the silencing assay, the cells were challenged with *P. gingivalis* (Pg) for 24 h. The expression levels of TLR4 (A) and TLR2 (B) were determined by quantitative PCR. (C) Production of IL-1β was measured by ELISA. The induction of the other cytokines (TNF-α [D], IL-6 [E], and IL-8 [F]) was determined in culture supernatants by Luminex 100 technology (Upstate Cell Signaling Solutions, NY). Data are presented as the means ± standard deviations (bars) of triplicate determinations. Statistically significant ($P < 0.05$) reduced gene expression or cytokine production is indicated by asterisks.

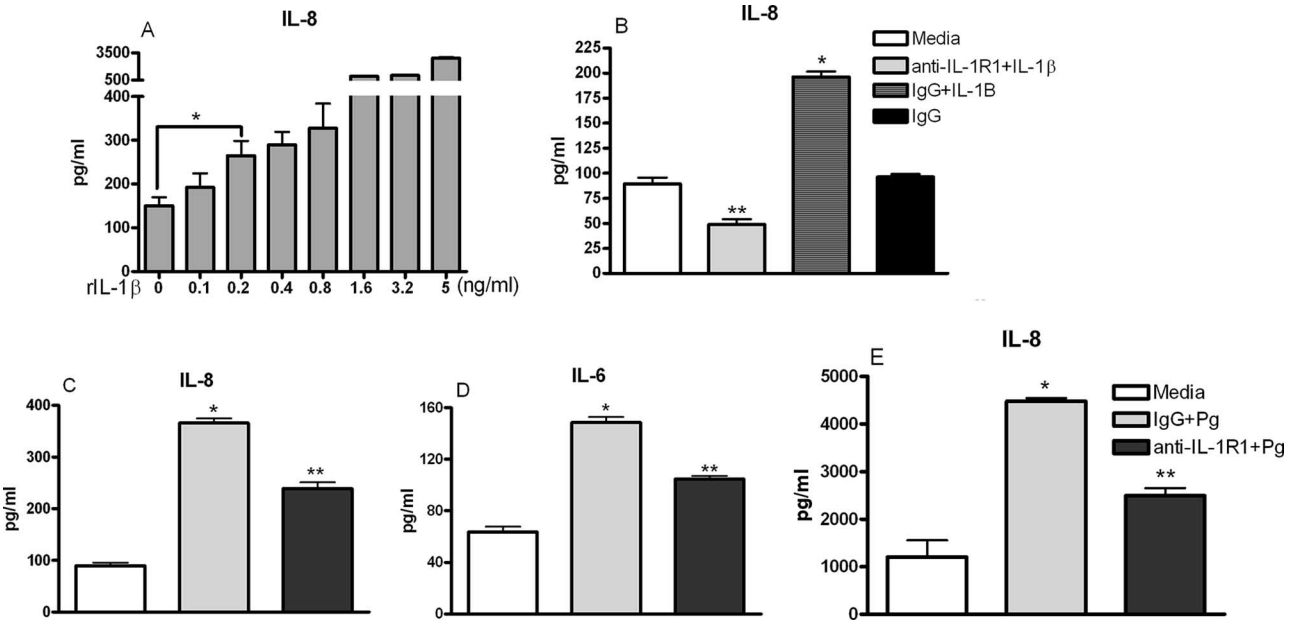

FIG. 6. Proinflammatory cytokine induction is IL-1β mediated in human epithelial cells. (A) TLR4 normal cells were challenged with 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, or 5 ng/ml of human recombinant IL-1β for 24 h, and then the production of IL-8 was determined by ELISA (BD Biosciences). (B) Human gingival epithelial cells were preincubated with an IL-1β receptor antagonist (1 μg/ml; IL-1R1) or its isotype control (IgG) for 1 h. Subsequently, the cells were challenged with exogenous IL-1β (0.2 ng/ml) for 24 h and then IL-8 production was measured by ELISA. (C) Human TLR4-normal gingival epithelial cells were preincubated with either anti-human IL-1R1 (1 μg/ml) or its isotype (IgG) for 1 h prior to the assay, and the cells were challenged with *P. gingivalis* (Pg) for 24 h at 37°C. Induction of IL-8 (C) and IL-6 (D) production was measured by ELISA. (E) In a manner similar to that described above, human bronchial epithelial cells were treated with *P. gingivalis* after blocking the IL-1β receptor (IL-1R1), and the induction of IL-8 was determined by ELISA. Data are presented as the means ± standard deviations (bars) of triplicate determinations. Statistically significant ($P < 0.05$) induction or reduction of cytokine release is indicated by single or double asterisks, respectively.

using siTLR2 did not reduce the production of proinflammatory cytokines, including IL-1β, IL-6, TNF-α, and IL-8, in HGECs following challenge with *P. gingivalis* (Fig. 5C to F). Therefore, our data clearly indicate that TLR4 plays a major role in upregulating proinflammatory cytokine induction in human primary epithelial cells treated with *P. gingivalis*.

Released IL-1β modulates proinflammatory cytokine induction in an autocrine manner in human epithelial cells. IL-1β is a potent proinflammatory cytokine produced following challenge with *P. gingivalis* (2), and it is considered to be a major player in the regulation of other inflammatory cytokines, such as IL-6 (10, 29). To examine IL-1β function, we initially confirmed that the IL-1β receptors were expressed by HGECs (data not shown). We determined that the optimal dose of IL-1β was similar to the amount of IL-1β produced by *P. gingivalis* (as shown in Fig. 5), which induces inflammation in the cells. We found that 0.2 ng/ml of human recombinant IL-1β significantly increased the induction of IL-8; however, 5 ng/ml of IL-1β maximized the induction of IL-8 (Fig. 6A). The cells were then challenged with 0.2 ng/ml of IL-1β in the presence or absence of anti-IL-1R1 antibody. We found that blocking the IL-1β receptor reduced the production of IL-8 (Fig. 6B) and the induction of other cytokines, such as IL-6 (data not shown). These data confirmed that IL-1β induced by *P. gingivalis* could regulate the "secondary" cytokine production in these cells. Further, the cells were subsequently challenged with *P. gingivalis* after blocking the IL-1β receptor to examine the effect of *P. gingivalis*-induced IL-1β. We demonstrate that the elevated IL-8 and IL-6 induction by *P. gingivalis* was attenuated when the IL-1β receptor was inhibited (Fig. 6C and D). We also found that IL-1β not only modulates the inflammation in gingival epithelial cells but also regulates the production of inflammatory cytokines, including IL-8, in human primary bronchial epithelial cells (Fig. 6E).

It has previously been shown that caspase-1 activation is a critical step in converting pro-IL-1β into mature IL-1β (20), and we have confirmed above that IL-1β plays an important role in the regulation of other inflammatory cytokines induced by *P. gingivalis* in HGECs. In order to examine these points further, we inhibited caspase-1 activation with a caspase-1 inhibitor, YVAD-cmk (26), and the cells were challenged with *P. gingivalis* in the presence or the absence of YVAD-cmk for 24 h. The production of IL-1β was dramatically reduced in the cells challenged with *P. gingivalis* after blocking caspase-1 activity (with YVAD-cmk) (Fig. 7A). Furthermore, we also observed that IL-6 (Fig. 7B) and IL-8 (Fig. 7C) production was reduced in epithelial cells challenged with *P. gingivalis* in the presence of YVAD-cmk. More importantly, we found that adding exogenous human recombinant IL-1β restored the production of cytokines, including IL-6, in the cells challenged with *P. gingivalis* after inhibiting caspase-1 activity with YVAD-cmk (Fig. 7D). Additionally, we showed that the production of IL-6, which was chosen as a representative cytokine, followed the induction of IL-1β. The induction of IL-1β increased within 30 min, but the production of IL-6 started to increase at 2 h. We then observed that the induction patterns of IL-1β and IL-6 followed a trend similar to that described above (Fig. 8). Taken together, our results clearly reveal that IL-1β modulates

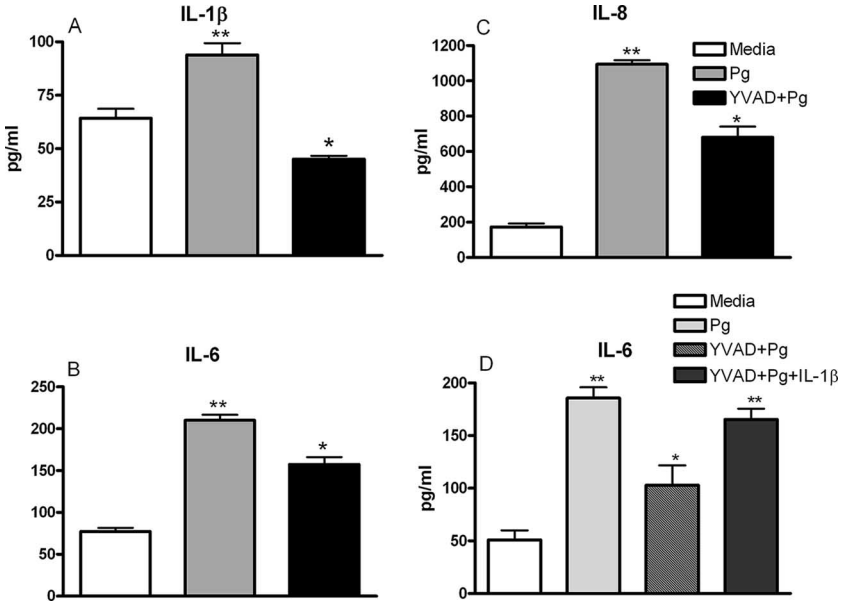

FIG. 7. Blocking IL-1β maturation reduced inflammation in epithelial cells. TLR4-normal cells were challenged with *P. gingivalis* for 24 h after blocking caspase-1. Caspase-1 was inhibited with YVAD-cmk (1 μM) 1 h prior to the assay in TLR4-normal cells challenged with *P. gingivalis* (Pg) for 24 h. IL-1β (A), IL-6 (B), and IL-8 (C) induction was measured by ELISA following the challenge assay. (D) The cells were challenged with *P. gingivalis* and IL-1β (0.2 ng/ml) after blocking caspase-1 with YVAD. Then, IL-6 induction was determined by ELISA (BD Biosciences). Data are presented as the means ± standard deviation (bars) of triplicate determinations. Statistically significant ($P < 0.05$) induction or reduction of cytokine production is indicated by single or double asterisks, respectively.

the proinflammatory cytokine induction in human epithelial cells following stimulation with *P. gingivalis*.

IL-8 does not modulate inflammation. We previously demonstrated that the level of IL-8 production is far greater than that of the other cytokines produced by epithelial cells (9). We therefore examined whether IL-8 could modulate the production of cytokines, including IL-1β and IL-6, in human epithelial cells. The cells were challenged with *P. gingivalis* in the presence of a neutralizing antibody to IL-8 or IgG, an isotype control for the antibody. The production of IL-8 was significantly increased by *P. gingivalis*, but the induction of IL-8 was undetectable in the cells challenged with *P. gingivalis* in the presence of its neutralizing antibody (Fig. 9A). In contrast, IL-1β (Fig. 9B) and IL-6 (Fig. 9C) production was significantly induced in the cells treated with *P. gingivalis* in the presence or the absence of the antibody. Taken together, our results demonstrated that IL-8 does not modulate the induction of IL-1β and IL-6.

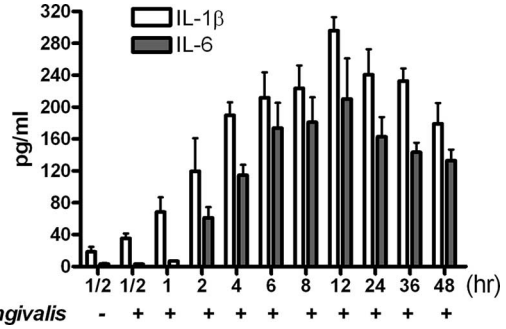

FIG. 8. IL-6 and IL-1β show similar trends. TLR4-normal cells were challenged with *P. gingivalis* (+) or medium only (−) at different time points, as indicated. The production of IL-1β and IL-6 was measured by ELISA (BD Biosciences). Error bars indicate standard deviations.

IL-1β can modulate inflammation in the cells following TLR activation. Finally, we examined whether cytokine production could be modulated by IL-1β in the cells challenged with a variety of other TLR-specific agonists. In a previous study, we have shown that HGECs express all TLRs except TLR8 (15). Therefore, HGECs were challenged with Pam3CSK4 (TLR1/TLR2 agonist), FSL-1 (TLR2/TLR6 agonist), or R837 (TLR7 agonist) in the presence or absence of a caspase-1 inhibitor, YVAD-cmk, for 24 h. The production of IL-1β was significantly induced by Pam3CSK4, FSL-1, or R837. However, the inhibition of caspase-1 activation resulted in reduced IL-1β induction following the challenge assay (Fig. 10A). We next examined whether this reduced IL-1β production was followed by reduced proinflammatory cytokine production as shown above. The induction of IL-6, a representative inflammatory cytokine, was significantly reduced in HGECs following challenge with Pam3CSK4, FSL-1, or R837 when caspase-1 activity was inhibited with YVAD-cmk (Fig. 10B). Our results showed that IL-1β production is a key step in modulating proinflammatory cytokine production in human epithelial cells.

DISCUSSION

Oral epithelial cells provide a physical barrier against invading pathogens and play an important role in the innate host defenses. Through the perturbation of gingival epithelial cells, *P. gingivalis* and other periodontal gram-negative bacteria can trigger several complex signaling cascades. These cascades ul-

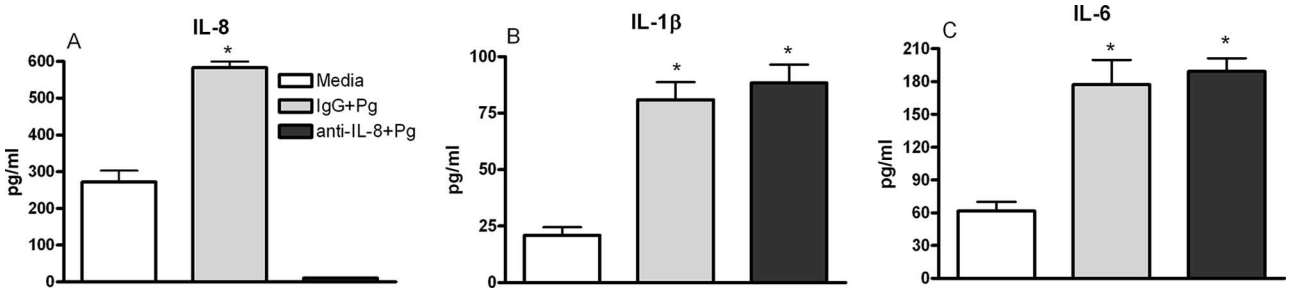

FIG. 9. IL-8 does not modulate cytokine production. TLR4-normal HGECs were challenged with *P. gingivalis* (Pg) in the presence of a neutralizing antibody for IL-8 or its isotype (IgG). The production of IL-8 (A), IL-1β (B), and IL-6 (C) was measured by ELISA. Data are presented as means ± standard deviations (bars). Statistically significant ($P < 0.05$) induction of IL-8, IL-1β, and IL-6 production is indicated by asterisks.

timately regulate the transcription of genes for proinflammatory cytokines, chemokines, antimicrobial peptides, apoptosis, and adaptive immune responses and might have a direct effect on disease and the inflammatory processes.

Epithelial cells are a first line of defense against pathogens, and although these cells are not as specialized as professional phagocytes in dealing with pathogens, they may play a sentinel role. The cells may use the extracellular secretion of IL-1β to induce other neighboring epithelial cells in an autocrine manner to help amplify the inflammatory, chemokine, and antimicrobial molecules released. Thus, it has quite different purposes and capabilities than the independently active professional phagocytes and, rather, may allow neighboring epithelial cells to act together in a concerted defense against periodontal microbes. *P. gingivalis* induces a strong proinflammatory cytokine response in gingival epithelial cells (15). We elected to utilize *P. gingivalis* in our experiments and to use primary epithelial cells in an attempt to mimic more closely the inflammatory in vivo situation relevant in chronic periodontitis. We observed that live *P. gingivalis* induced apoptosis in HGECs, and at the end of the challenge assay, the cell viability was less than 50% (data not shown). Therefore, the use of heat-killed bacteria allowed the examination of secondary cytokine induction by *P. gingivalis.* Although other periodontal gram-negative microorganisms are present and important in the periodontal plaque biofilm, we sought to represent periodontal microbial challenge to the periodontal pocket epithelial lining by using heat-killed *P. gingivalis*. The present data demonstrate that TLR4 plays an important role in the regulation of IL-1β in the cells challenged with *P. gingivalis*.

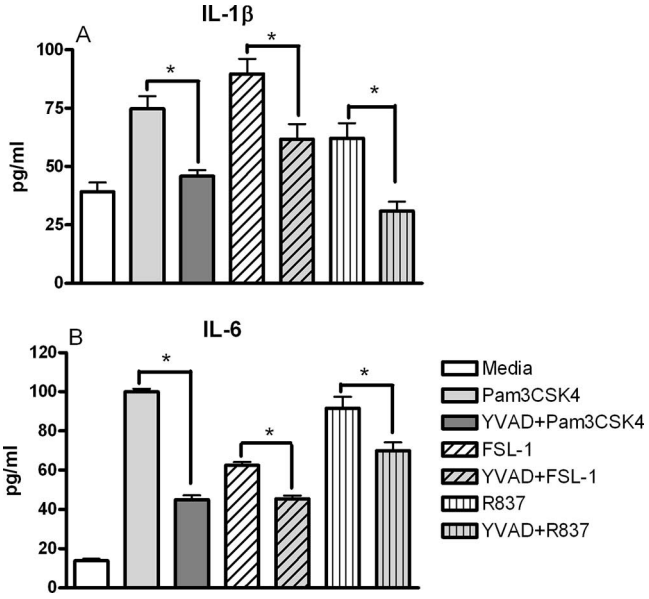

FIG. 10. TLR2- and TLR7-induced IL-6 is mediated by IL-1β. Caspase-1 activation was inhibited with YVAD-cmk (1 μM) prior to 1 h of the challenge assay. HGECs were then challenged with TLR1/TLR2 agonist (Pam3CSK4), TLR2/TLR6 agonist (FSL-1), and TLR7 agonist (R837) for 24 h. IL-1β (A) and IL-6 (B) induction was measured by ELISA following the challenge assay. Data are presented as the means ± standard deviations (bars) of triplicate determinations. Statistically significant ($P < 0.05$) reduction of IL-1β and IL-6 production is indicated by asterisks.

IL-1β is one of the most potent inflammatory cytokines, and its induction is tightly controlled. Typically, the production of IL-1β is regulated by at least two steps, the first at the transcription level by TLRs and the second at the posttranscriptional level by the inflammasome, which is a protein complex activating caspase-1 (25). Interestingly, it has been demonstrated that the activation of caspase-1 is TLR independent (13). We consistently found that IL-1β expression at the gene level (Fig. 2A) was not induced in TLR4-deficient cells treated with *P. gingivalis*, indicating that the reduced IL-1β production is due to reduced TLR4 signaling. IL-1β secretion is induced soon after microbial invasion (27), so one could hypothesize that IL-1β might play an important role in the induction of other inflammatory cytokines. Our results showed not only that the induction of IL-1β was reduced but also that the induction of other cytokines, including IL-8, IL-6, GM-CSF, and TNF-α, was reduced in TLR4-deficient cells treated with *P. gingivalis* (Fig. 3). Nevertheless, TLR4-deficient cells showed a normal response to FSL-1 or IL-1β (Fig. 4), indicating that the cells can be activated by other stimuli. We demonstrated that reducing TLR4 expression in TLR4-normal cells resulted in reduced cytokine production following challenge with *P. gingivalis*; however, reducing TLR2 expression did not result in reduced cytokines, suggesting that TLR4 is mostly involved in *P. gingivalis* recognition (Fig. 5). Interestingly, when we reduced the TLR4 expression in TLR4-normal cells, the cytokine production was not completely abrogated. This result is explained by the fact that other receptors, including RP105, are

involved in *P. gingivalis* recognition, since it has previously been shown that RP105 can recognize lipopolysaccharide in addition to TLR4 (14), and we also found that RP105 is expressed by epithelial cells and can be induced by *P. gingivalis* (data not shown).

Furthermore, our study clearly shows that by inhibiting caspase-1 activation or the IL-1β receptor with YVAD-cmk or IL-1R1 antibody, respectively, the induction of other proinflammatory cytokines in the cells following treatment with *P. gingivalis* was reduced. We found that *P. gingivalis* can induce 0.2 ng/ml of IL-1β in the cells, and adding the same amount of IL-1β to the cell cultures could increase the production of cytokines (Fig. 6A and B), indicating that *P. gingivalis*-induced IL-1β has the ability to modulate the production of cytokines. Alternatively, blocking the IL-1β receptor reduced the induction of cytokines in the cells challenged with *P. gingivalis* (Fig. 6C and D). Our results also showed that the production of cytokines was rescued in the cells challenged with *P. gingivalis* in the presence of both YVAD and recombinant IL-1β (Fig. 7D), indicating that IL-1β plays a major role in modulating the production of other cytokines, such as IL-6 and IL-8. Moreover, we observed that at the indicated time point, in epithelial cells treated with *P. gingivalis*, IL-1β and IL-6 induction showed trends similar to those described above (Fig. 8). These findings clearly reveal that proinflammatory cytokine production is IL-1β modulated in human epithelial cells. However, we observed that neither IL-1β nor IL-6 could be mediated by *P. gingivalis* induction of IL-8 (Fig. 9). Furthermore, our results reveal that inflammatory cytokine production induced by the activation of a variety of TLRs, in addition to TLR2 and TLR4, is IL-1β mediated in human epithelial cells (Fig. 10). Together, these data indicate that TLR4, in addition to other TLRs, plays an important role in the induction of IL-1β, and subsequently, IL-1β modulates the induction of other proinflammatory cytokines in human primary epithelial cells.

In summary, the work presented here supports a model whereby the regulation of secondary proinflammatory cytokine production in epithelial cells is modulated by IL-1β. This result may be important for the gingival crevice, the site where periodontal inflammation is initiated, as *P. gingivalis*, a representative of the predominantly gram-negative subgingival plaque biofilm, induces IL-1β, which modulates the induction of other proinflammatory cytokines in gingival epithelial cells. Inflammation so initiated then clinically manifests as gingivitis and subsequently as chronic periodontitis. In a more general context, the regulation of IL-1β production may be an effective therapeutic target for the modulation of inflammation in the treatment of such inflammatory diseases as gout or rheumatoid arthritis (3, 22).

ACKNOWLEDGMENT

This study was supported by grant NIH NIDCR DE017384.

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Editor: B. A. McCormick